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Prevalence of TTV DNA Among Children With a History of Transfusion or Liver Disease

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The prevalence rates of serum TT virus (TTV) DNA among children with or without a history of transfusion or liver disease were studied by polymerase chain reaction (PCR) using either the Okamoto primer set or the Takahashi primer set developed more recently. Using Okamoto and Takahashi primer sets, the prevalence rates were 31.6% (12/38) and 78.9% (30/38), respectively, for children with a history of blood transfusion (including malignant and non-malignant groups) and 6.7% (2/30) and 60% (18/30), respectively, for children without a history of blood transfusion. Among pregnant women, these rates were 12.9% (4/31) and 61.3% (19/31), respectively. On the other hand, the prevalence rates were 0% (0/16) and 50% (8/16), respectively, in hepatitis B patients, 21.4% (3/14) and 71.4% (10/14), respectively, for hepatitis C patients, and 20.0% (9/45) and 57.8% (26/45), respectively, for non-A to C hepatitis patients (including 27 acute hepatitis patients, 5 fulminant patients and 13 chronic hepatitis patients). In this study, the prevalence rates determined by the Takahashi primer set tended to be 2–9 times higher than those determined using the Okamoto primer set. These results suggest that TTV infection is widespread among Japanese children. Furthermore, blood transfusion does not appear to be the major route of infection. The similar prevalence rates between control children and children with various types of hepatitis using the Takahashi primer system suggest that TTV infection does not play a direct causative role in the development of liver disease in children. ***J. Med. Virol. 60:172–176, 2000.***

KEY WORDS: hepatitis; novel viral agent; PCR; TT virus

INTRODUCTION

In 1997, a new viral agent, TT virus (TTV) was discovered in the serum of posttransfusion hepatitis patients with unknown etiology [Nishizawa et al., 1997]. TTV has been found in the serum of 41% of patients with non-A to E chronic liver disease, but in only 12% of blood donors in Japan [Okamoto et al., 1998a]. Consequently, this virus has been implicated as a cause of non-A to E hepatitis. This virus has been found in patients with liver disorders in many countries [Charlton et al., 1998; Höhne et al., 1998; Mushahwar et al., 1999; Naoumov et al., 1998; Simmonds et al., 1998; Tanaka et al., 1998]. However, to date the pathological role of TTV infection in liver disease remains unclear.

A recent study using a new polymerase chain reaction (PCR) primer indicated that this virus has a prevalence of 92% in the general adult population in Japan, suggesting that it does not play such a large role in the development of liver disease [Takahashi et al., 1998]. To date, these studies have targeted exclusively adults, so no data are available for children. The detection rate of serum TTV DNA by PCR among children with a history of blood transfusion or hepatic disorder was investigated.

MATERIALS AND METHODS

Initially, 30 children (control children) and 31 pregnant women were examined. The underlying disease in these children was respiratory tract infection, digestive tract infection, or bronchial asthma, and none had a history of blood transfusion (including blood products) or liver disease. Similarly, none of the pregnant women had a history of blood transfusion or liver disease.

Subsequently, thirty-eight children with a history of blood transfusion were examined. These children were divided into a malignant group and a non-malignant group. The malignant group consisted of 21 children who had been given antineoplastic agents, including immunosupressive drugs for the treatment of leukemia or solid tumors. The non-malignant group consisted of 17 patients who had a history of transfusions for non-malignant diseases, including surgery for congenital

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heart disease, premature delivery, and severe infection. At least 1 month had passed between the first transfusion and blood sampling. The number of blood-donor exposures was 41.1 ± 36.1 in the malignant group and 20 ± 48.6 in the non-malignant group. The malignant group included 4 children with hepatitis C virus (HCV) and the non-malignant group included 6.

Finally, 75 children who had an ALT level over 40 IU/l were examined. These children were divided into the following groups according to type of liver disease: chronic hepatitis B (hepatitis B) group, chronic hepatitis C (hepatitis C) group, and sporadic non-A, non-B and non-C (non-A to C) acute, fulminant or chronic hepatitis group. Five patients of the hepatitis B group and 7 patients of the hepatitis C group had been infected by horizontal transmission. The remaining children in the two groups were infected by mother-to-infant transmission. The non-A to C hepatitis group consisted of 45 patients, 27 of whom had acute hepatitis, 5 who had fulminant hepatitis and 13 who had chronic hepatitis. None of the hepatitis B or non-A to C hepatitis patients had a history of blood transfusion.

Serum samples obtained from all patients were stored at –30°C until use. Parental consent was obtained for blood sampling in children.

DNA was extracted from 100 μl of each patient's serum using the phenol-chroloform-isoamyl alcohol procedure as previously described [Miyake et al., 1996]. Using the isolated DNA, PCR was performed using primers synthesized according to the published TTV sequence by Okamoto et al. [1998a] and by Takahashi et al. [1998]. With the Okamoto primer system, semi-nested PCR was carried out and the sense primer sequences used for 1st and 2nd round PCR were 5′-ACAGACAGAGGAGAAGGCAACATG-3′and 5′-GGCAACATGTTATGGATAGACTGG-3′, respectively, and the antisense primer sequence was 5′-CTGGCATTTTACCATTTCCAAAGTT-3′. With the Takahashi primer system, double PCR was carried out and the sense and antisense primers used for 1st and 2nd round PCR were 5′-GCTACGTCACTAACCAC-GTG-3′ and 5′-CTBCGGTGTGTAAACTCACC-3′ (B = mixture of G, C, and T). The first PCR was carried out on 20 (μl aliquots of sample using Taq polymerase (Takara Shuzou, Otsu, Japan) for 35 cycles, each of which consisted of denaturation for 1 min at 94°C, annealing for 1 min at 55°C, and extension for 2 min at 72 °C (with an additional 7 min extension during the last cycle). The second PCR was carried out for 30 cycles under the same conditions using 2 μl of a 10X-diluted solution of 1st round PCR products. PCR products were separated by electrophoresis on 3% agarose gels, stained with ethidium bromide, then observed under UV light. Sera were determined to be TTV DNA-positive based on the presence of a 271 bp band in the Okamoto primer system and a 199 bp band in the Takahashi primer system. All reagents were examined for TTV DNA contamination, and all experiments were conducted in parallel with positive and negative control sera.

To determine the sensitivity of the Takahashi primer system, serum samples obtained from 8 TTV-positive subjects determined by Okamoto primer system were used as a standard. Prior to PCR, 2 μl of DNA extracted from these serum samples was diluted from 1 to 10^{-5} times with distilled water and PCR was carried out using the two primer systems described above. In order to examine the specificity of Takahashi primer system, the nucleotide sequences of PCR products obtained from 30 TTV-positive subjects were analyzed. The amplified DNA fragments were purified using the QIAquick Gel Extraction Kit (QIAGEN k.k. Tokyo, Japan). The purified DNA were sequenced by the dideoxy-mediated chain-termination method, using a BigDye Primer Cycle Sequencing, FS Ready Reaction Kit (Applied Biosystems, Chiba, Japan) and a ABI PRISM™ 310 Genetic Analyser (Applied Biosystems, Chiba, Japan) according to the manufacturers' instructions. In the sequencing reaction, the antisense primer for double PCR was used as the sequencing template. The 100 nucleotides (nt26–nt125) were compared to those of the consensus sequences of GenBank (accession number; AB 008394, version; AB 008394.1).

Statistical analysis was carried out using Welch's t-test or Fisher's exact probability test.

RESULTS

The sensitivity of PCR using Okamoto and Takahashi primer systems was determined. Using these systems, the titers of the end-point dilution that gave a positive result was 1 and 1 for 1 sample, 1 and 10^{-1} for 2 samples, 10^{-2} and 10^{-2} for 2 samples, 10^{-2} and 10^{-3} for 1 sample, and 10^{-3} and 10^{-3} for 2 sample, respectively. These results indicate that overall, the two systems provide similar sensitivity, although Takahashi primer system tended to show greater sensitivity in some samples.

The frequency of homology in the 100 nucleotides (nt26-nt125) between each sample obtained from 30 TTV-positive subjects according to the Takahashi primer system and that of accession TTV in GenBank (AB 008394.1) ranged between 71% to 86% (mean ± standard deviation; 80.1 (4.9%). Subsequent homology searches in GenBank revealed that all 30 nucleotide sequences were most homologous to those of TTVs.

Table I shows the prevalence rates of serum TTV DNA in children with and without a history of blood transfusion and in pregnant women. The prevalence rates using the Okamoto primer system was 31.6% (12/38) for the transfused group (including malignant and non-malignant groups; the rates were 28.6% (6/21) and 35.3% (6/17), respectively), 6.7% (2/30) for the control group and 12.9% (4/31) for the pregnant women. On the other hand, the rates using the Takahashi primer system were 78.9% (30/38), 76.2% (16/21), 82.4% (14/17), 60% (18/30) and 61.3% (19/31), respectively. All serum TTV DNA-positive cases with the Okamoto primer sys-

TABLE I. Prevalence of Serum TTV DNA

	Number of cases (male; female)	Age (years)	ALT (IU/l)	Prevalence of TTV DNA: Okamoto primer system (%)	Takahashi primer system (%)	Both primer systems (%)
Children with a history of transfusion	38 (25; 13)	6.9 ± 5.7	89 ± 154	12/38 (31.6)	30/38 (78.9)	30/38 (78.9)
Malignant	21 (16; 5)	7.8 ± 6.5	42 ± 70	6/21 (28.6)	16/21 (76.2)	16/21 (76.2)
Non-malignant	17 (9; 8)	5.7 ± 4.3	116 ± 200	6/17 (35.3)	14/17 (82.4)	14/17 (82.4)
Children without a history of transfusion	30 (20; 10)	5.0 ± 4.9	18 ± 8	2/30 (6.7)	18/30 (60.0)	18/30 (60.0)
Pregnant women without a history of transfusion	31	28.4 ± 10.5	22 ± 10	4/31 (12.9)	19/31 (61.3)	19/31 (61.3)

Age, mean ± standard deviation (years); ALT, alanine amino transferase (IU/l); Statistical analysis was performed using Fisher's exact probability test.
*$P < 0.05$; **$P < 0.01$.

tem were positive for serum TTV DNA with the Takahashi primer system. The Takahashi primer system indicated a prevalence rate of 2 to 9 times higher than that determined by the Okamoto primer system. No significant differences were observed with respect to sex ratio, mean age, rate of HCV infected patients, number of blood-donor exposures (the number of exposures to blood-donors was almost identical to the number of transfusions) ($P > 0.05$). Significant differences were observed in the prevalence rate between the malignant and non-malignant groups including the transfusion group and the control group, according to the Okamoto primer system. However, these differences were not observed with the Takahashi primer system.

Table II shows the prevalence rates in groups with various types of liver disease. Of the 75 children with liver disease, 2 non-A to C acute hepatitis patients had serum TTV DNA-positivity only with the Okamoto primer system. Overall, the prevalence rates were 0% (0/16) for the hepatitis B group, 21.4% (3/14) for the hepatitis C group, 20% (9/45) for the all non-A to C hepatitis group, 22.2% (6/27) for the non-A to C acute hepatitis group, 40% (2/5) for the non-A to C fulminant hepatitis group and 7.7% (1/13) for the non-A to C chronic hepatitis group with the Okamoto primer system. In contrast, these rates with the Takahashi primer system were 50% (8/16), 71.4% (10/14), 57.8% (26/45), 44.4% (12/27), 80% (4/5) and 76.9% (10/13), respectively. The average ALT level and the prevalence rates of serum TTV DNA in both the primer systems, was higher for the fulminant hepatitis group. No significant differences in prevalence rate were found between the groups with various types of liver disease and the control group using either primer system. In addition, average ALT levels between TTV DNA-positive and-negative cases in various types of liver diseases did not differ in the current study (data not shown).

DISCUSSION

Takahashi et al. [1998] reported that the sensitivity of the PCR system using Takahashi's primers to detect TTV DNA was superior to that using Okamoto's primers by 10–100 fold in a single-step 55-cycle PCR using Ampli-Taq Gold. However, we found the system to be only 1–10 fold more sensitive than the Okamoto primer system. One possible reason for this difference is that we used a double PCR in both primer systems. However, reamplification using the same primers used in the Takahashi primer system appears to be a procedure with an increased risk of contamination. Therefore, all experiments were carried out in parallel with negative control samples to eliminate contamination. Although all TTV DNA-positive samples according to the Takahashi primer system were not analyzed, the nucleotide sequences of 30 samples analyzed were homologous to those of accession TTV sequences in GenBank. Thus, it is considered that the current study using the Takahashi primer system was specific to the detection of TTV DNA.

The prevalence rates in the control group and in the pregnant women group using the Okamoto primer system, were similar to those of a previous report [Goto et al., 1999], blood donors in Japan [Okamoto et al., 1998a], and healthy controls in the UK [Naoumov et al., 1998] and North America [Charlton et al., 1998]. TTV has been considered a virus transmitted by blood due to its high detection rate in individuals with a history of blood transfusion using the Okamoto primer system [Naoumov et al., 1998, Okamoto et al., 1998a, Simmonds et al., 1998]. Our results using the Okamoto primer system suggest that this is also the case in children.

However, a PCR primer system developed recently, the Takahashi primer system, provided evidence that contradicts that of the Okamoto primer system, since it suggests a very high prevalence rate (92%) of TTV infection in the general adult population in Japan. We applied this system to various child populations and compared the results to those obtained using the Okamoto primer system. It was found that the prevalence rate of the transfused group did not differ from that of the control group according to the Takahashi primer system, suggesting that TTV infection occurs widely

TABLE II. Prevalence of Serum TTV DNA in Children With Various Types of Liver Disease

	Number of cases (male; female)	Age (years)	ALT (IU/l)	Prevalence of TTV DNA: Okamoto primer system (%)	Takahashi primer system (%)	Both primer systems (%)
Hepatitis B	16 (9; 7)	7.3 ± 4.2	415 ± 389	0/16 (0.0)	8/16 (50.0)	8/16 (50.0)
Hepatitis C	14 (5; 9)	8.2 ± 4.8	129 ± 195	3/14 (21.4) *	10/14 (71.4)	10/14 (71.4)
Non-A to C hepatitis	45 (22; 23)	4.4 ± 5.1	523 ± 654	9/45 (20.0) *	26/45 (57.8)	28/45 (62.2)
Acute hepatitis	27 (11; 16)	3.7 ± 5.5	342 ± 536	6/27 (22.2)	12/27 (44.4)	14/27 (51.9)
Fulminant hepatitis	5 (1; 4)	7.7 ± 3.5	1484 ± 475	2/5 (40.0)	4/5 (80.0)	4/5 (80.0)
Chronic hepatitis	13 (10; 3)	4.7 ± 4.6	531 ± 642	1/13 (7.7)	10/13 (76.9)	10/13 (76.9)

among Japanese children. Thus, blood transfusion does not appear to be the major route of TTV infection. Okamoto et al. [1998b] proposed fecal-oral transmission as a possible route for TTV infection. The transmission route of TTV in the present study remains unclear.

The prevalence rate of serum TTV DNA in the control group and in the pregnant women using the Takahashi primer system was remarkably lower than the 92% reported in the general adult population [Takahashi et al., 1998]. The reasons for this difference are not clear, but this may represent an age-related difference.

In the initial report on the Takahashi primer system, all serum TTV DNA-positive cases using the Okamoto primer system were serum TTV DNA-positive with the Takahashi primer system. In contrast, in a few cases, serum TTV-DNA positivity was detected only in the Okamoto primer system. This is likely due to the low amplification efficiency of the Takahashi primers on structural changes of the target template DNA in such cases.

The pathological role of TTV infection in liver disease in adults remains unclear. There are currently two schools of thought: one that believes TTV represents a candidate virus that may explain the cause of hepatitis [Höhne et al., 1998, Ikeda et al., 1999, Nishizawa et al., 1998, Okamoto et al., 1998a, Tanaka et al., 1998] and the other that considers that it is unrelated [Charlton et al., 1998, Naoumov et al., 1998, Simmonds et al., 1998, Takahashi et al., 1998]. In the present study, the higher prevalence rate of serum TTV DNA among non-A to C acute and fulminant hepatitis patients compared to the hepatitis B group using the Okamoto primer system cannot be easily explained. In contrast, the prevalence rate of serum TTV DNA among non-A to C acute and fulminant hepatitis patients was slightly higher than in compared to the control group using the Okamoto primer system were found but this difference was not significant ($P = 0.095$, $P = 0.089$). Furthermore, the overall lack of significant differences in the prevalence rates with the Takahashi primer system between children with various types of hepatitis and the control group indicates that liver disease does not result directly from TTV infection in children. Therefore, TTV should not be considered a causative agent of liver diseases of unknown etiology, nor does it affect the degree of liver damage when present in co-infection with HBV or HCV. However, these results do not negate the pathogenecity of TTV in which liver disease may develop later and further studies are necessary.

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